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RAPD Fingerprinting and Demonstration of Genetic Variation in Three Pathogens Isolated from Mangrove Environment

[1]M. Thangaraj, [1]Vishruth Prem, [1]T. Ramesh and [2]A.P. Lipton
[1]Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, 608 502, India
[2]VRC of CMFRI, Vizhinjam, Kerala, India

Corresponding Author: M. Thangaraj, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, 608 502, India

ABSTRACT

The aim of the study was to find the RAPD finger printing of *Pseudomonas aeruginosa*, *Bacillus circulans* and *Serratia marcescens,* using ten random primers. The RAPD-PCR produced reproducible electrophoretic band patterns only in seven primers. There were 67 species specific bands observed, among these, 25 bands in *P. aeruginosa,* 19 in *B. circulans* and 23 in *S. marcescens*. This study suggests that RAPD typing could be an additional rapid typing method for studying the epidemiology of these pathogens. Based on the RAPD data the genetic identity between *S. marcescens* and *B. circulans* was more (0.3803) than the other.

Key words: *Pseudomonas aeruginosa, Bacillus circulans, Serratia marcescens,* RAPD, PCR

INTRODUCTION

Pseudomonas aeruginosa is considered as an opportunistic pathogen as it causes infection in immunodepressed subjects or in those with faulty homeostasis mechanisms (Walker, 1998; Kiska and Gilligan, 1999). It causes a variety of infections (e.g., urinary tract infections, respiratory system infections, dermatitis, soft tissue infections and gastrointestinal infections) with a major problem for people hospitalized with cancer, cystic fibrosis and burns (Rowe *et al*., 2005; Driscoll *et al*., 2007). *Serratia marcescens* occurs in human clinical specimens, soil, water, plant surfaces and other environmental sites, digestive tract of rodents and insects.

The DNA typing can be done in two methods. The first is based on restriction digestion and the second is based on PCR method. In the first method the DNA is subjected to restriction enzymes which cut the DNA at specific sites and produce a DNA pattern called the fingerprint, which will be unique for every individual. In the second method, PCR (Polymerase Chain Reaction) is made use of and produce thousands of copies of specific DNA sequence. This allows comparing the DNA sequences and also the presence or absence of a small DNA sequence in the whole genome (Van Belkum, 1994). Random amplified polymorphic DNA (RAPD) analysis is a technique based on the Polymerase Chain Reaction (PCR) amplification of discrete regions of genome with short oligonucleotide primers of arbitrary sequence (Welsh and McClelland, 1990; Williams *et al*., 1990). Shi and Xia (2005) studied the genetic variation in clones of *Pseudomonas pseudoalcaligenes* using RAPD method. Quednau *et al*. (1997) used the same technique for rapid, reliable and easily identification of clinically important species of *Enterococcus* within a day. The method is simple and rapid method for determining genetic diversity and no prior of the genome under study is required

(Hadrys *et al*., 1992). RAPD analysis also has been used to evaluate genetic diversity and to discriminate different strains like *Pseudomonas aeruginosa* (Ortiz-Herrera *et al*., 2004), *Vibrio parahaemolyticus* and *V. alginolyticus* (Sudheesh *et al*., 2002), *Acanthamoeba divineness* (Ortega-Rivas *et al*., 2003), *Pseudomonas pseudoalcaligenes* (Shi and Xia, 2005).

The mangrove environment possesses a variety of microorganisms which are having extreme adaptation to tolerate salinity and temperature fluctuations. In this microbial community, majority are pathogenic to both animals and human. Among this mangrove pathogens, *Pseudomonas aeruginosa*, *Bacillus circulans* and *Serratia marcescens* are the dominant microbial community present in this study area oftenly causes disease to fishes. Therefore, in this study RAPD was adopted to probe the fingerprinting and potential genomic difference and it could be an additional rapid typing method for studying the epidemiology of these pathogens.

MATERIALS AND METHODS

Isolation of bacteria: Sediment samples were collected during December, 2009 in sterile glass wares from the mangrove area of Vellar estuary and brought to laboratory. One gram of sediment was weighed accurately and mixed in 99 mL of sterile distilled water. Serial dilutions were made from this mixture to get different concentrations. From the diluted sample, 0.1 mL was spread into petriplates containing nutrient agar for enumeration of total bacterial population. The plates were incubated at 37°C for 24 h following which the isolated colonies were picked for pure culture by streak plate method.

Species identification: By using morphological characters, colour formation and biochemical tests, three isolates were identified as *Pseudomonas aeruginosa, Bacillus circulans* and *Serratia marcescens*.

Genomic DNA extraction: The DNA extraction method we used was as follows: The bacterial cells were harvested by centrifugation at 500 g for 10 min, 4°C. The cell pellet was washed three times with cold Phosphate Buffered Saline (PBS) and was incubated with lysis solution (EDTA, 0.5 M; Sarcosyl, 10 g L^{-1}, Kcl, 100 mM; Tris, 40 mM; $MgCl_2$, 5 mM; Tween-20, 1%) and 0.25 mg mL^{-1} proteinase K. Tubes were incubated at 56°C for 4 h. A volume of TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1) was added to DNA sample and the mixer was centrifuged at 2000 g for 5 min at room temperature to separate the phases. The aqueous layer was removed to a clean tube and DNA was precipitated using chilled sodium acetate (3 M) and isopropanol. Finally, the pellet was washed with ethanol (70%) to remove the excess salt.

PCR reactions and product analysis: A total of 10 decameric oligonucleotides of arbitrary sequence primers were used. According to the number and intensity of the resulting bands, the reproducibility and discriminating potential of the amplified products, seven primers were selected for further analyses. The DNA amplification reactions were performed as Williams *et al*. (1990) describes, in 25 µL volumes containing 10 ng template DNA, Buffer KCl, 50 mM; Tris-HCl, 10 mM; MgCl2, 2.5 mM, pH 8.5; dNTP, 200 mM; primer, 0.6 mM and 0.4 units of *Taq* DNA Polymerase (Genei, Bangalore) in a Tech gene thermocycler. The cycling conditions were: an initial denaturing phase of 94°C for 3 min and 40 repetitions at 94°C for 1 min, 35°C for 1 min and 72°C for 1.5 min. The final extension phase was prolonged for 10 min at 72°C in the last cycle. Amplification products were fractionated by 2% agarose electrophoresis, stained with a solution of 0.5 µg mL of ethidium bromide and visualized under UV light.

Genetic similarity/distance between three bacterial species was estimated using PopGene Software (Yeh *et al.,* 1999). Nei and Li (1979) Genetic Similarity (GS) among three bacterial species was computed and converted by PopGene into Genetic Distance (GD) according to Hillis *et al.* (1996) formula, GD = 1-GS.

Phylogenetic relationship based on genetic distance values generated from RAPD data among three bacterial species was made and dendrogram plotted, following unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) based on Nei (1978) modified from NEIGHBOUR procedure of PYLIP version 3.5 c (Felsenstein, 1993) using PopGene version 1.31 (Yeh *et al.*, 1999).

RESULTS

Private bands (species specific markers): There were 75 bands were generated with nine random primers in three bacterial species (Fig. 1). Out of these 67 were species specific or private bands. Twenty five private alleles in *P. aeruginosa,* ninteen private alleles in *B. circulans* and twenty-three private alleles in *S. marcescens* were observed.

Genetic similarity and distance: Nei (1978) unbiased genetic distances and genetic similarity between three bacterial species are given in Table 1, the genetic distance between *P. aeruginosa* and *B. circulans* was 1.086. The genetic distance between *P. aeruginosa* and *S. marcescens* was 1.2669. The genetic distance between *B. circulans* and *S. marcescens* was 0.9668. The genetic identity between *S. marcescens* and *B. circulans* was more (0.3803) than the other.

Dendrogram: Based on Nei's genetic distance value a neighbour joining tree (1000 replications) was generated by MEGA (Version 4.2) and depict in Fig. 2.

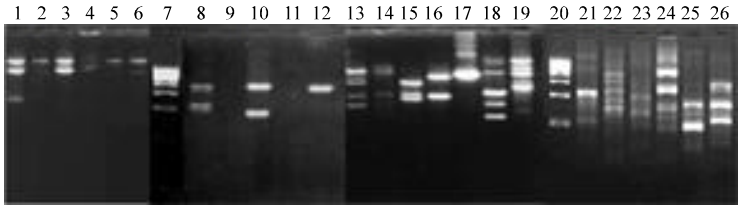

Fig. 1: Electrophoretic pattern generated by arbitrary primers (*Ba1- Ba7*) in three bacterial species. Lanes 7, 13 and 20: 500-bp DNA ladder. Lanes 1, 4, 8, 14, 17, 21 and 24: *Pseudomonas aeruginosa*. Lanes 2, 5, 10, 15, 18, 22 & 25: *Bacillus circulans*. Lanes 3, 6, 12, 16, 19, 23 and 26: *Serratia marcescens*

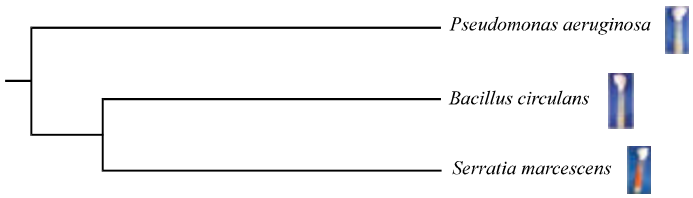

Fig. 2: Neighbour joining tree (1000 replications) generated by MEGA (Version 4.2) from RAPD data of three bacterial species. Scale indicates the genetic distance

Table 1: Nei (1978) genetic identity (above diagonal) and genetic distance (below diagonal) of three bacterial species

	Pseudomonas aeruginosa	*Bacillus circulans*	*Serratia marcescens*
Pseudomonas aeruginosa	*****	0.3380	0.2817
Bacillus circulans	1.0846	*****	0.3803
Serratia marcescens	1.2669	0.9668	******

DISCUSSION

The samples were collected from the natural environment and so the three species were separated from mixed culture. Due to the colour variation in nutrient agar medium it was very easy to differentiate each other. In general, molecular methods combine higher discriminatory power and higher reproducibility than phenotypic tests. These advantages are a result of their ability to detect minor genome differences and the higher stability of molecular targets compared with that of phenotypic profiles for some species (Tenover *et al*., 1997). However, for *V. harveyi*-related species, highly similar genomes and genome plasticity may also limit precise identification by molecular techniques in some cases (Thompson and Swings, 2006; Sawabe *et al*., 2007). A range of different molecular methods have been used for identification and typing of bacterial species. The discriminatory power of different molecular methods varies widely, as does the ease and speed of performance and cost. The decision to use a molecular technique, as opposed to a particular phenotypic method, should be based on a comparison of simplicity, necessity for high throughput analysis and cost (Riley, 2004). A number of DNA fingerprinting methods are followed for many bacterial species identification (Pujalte *et al*., 2003; Hernandez and Olmos, 2004; Alavandi *et al*., 2006; Musa *et al*., 2008).

The most widespread of the several RAPD variants envisages PCR with only one primer with a random sequence of 9-11 nucleotides in length. The optimal primer cannot be constructed in silico and is chosen from a series of analogous primers by testing. Primers that give more amplification products and, accordingly, more bands on the fingerprints are usually chosen. Thus, in one of the studies on employment of RAPD for identification of bifidobacteria, only seven 10-nucleotide primers were selected out of the 80 tested (Fanedl *et al*., 1998). In this present study seven primers were got amplified out of ten primers tried. The disadvantage of RAPD is poor reproducibility of fingerprints. RAPD needs strict standardization of PCR conditions, because the use of different polymerases or DNA/primer ratios or different annealing temperatures may lead to a discrepancy in the results obtained at different laboratories or in different time periods with the same samplings of strains. The ERIC fingerprints were unique for each species but similar for closely related species, e.g., for *B. catenulatum* and *B. pseudocatenulatum* (Ventura *et al*., 2003). Likewise in this study *Pseudomonas aeruginosa*, *Bacillus circulans* and *Serratia marcescens* have their own RAPD finger prints but *B. circulans* and *S. marcescens* shares some bands and showed these are closely related species.

CONCLUSION

The common RAPD bands, shared between these two genera, may allow the development of genus specific DNA markers for *Serratia and Bacillus,* which will facilitate identification of these two pathogens by multiplex analysis. This will be a valuable tool for epidemiological studies of these bacteria and important for the development of selective pathogen management strategies. Further studies with other molecular methodologies are essential to clarify and confirm the finger print and genetic relationships among the microbial flora in the mangrove environment.

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REFERENCES

Alavandi, S.V., V. Manoranjita, K.K. Vijayan, N. Kalaimani and T.C. Santiago, 2006. Phenotypic and molecular typing of *Vibrio harveyi* isolates and their pathogenicity to tiger shrimp larvae. Lett. Applied Microbiol., 43: 566-570.

Driscoll, J.A., S.L. Brody and M.H. Kollef, 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs, 67: 351-368.

Fanedl, L., F.V. Nekrep and G. Avgustin, 1998. Random amplified polymorphic DNA analysis and demonstration of genetic variability among Bifidobacteria isolated from rats fed with raw kidney beans. Can. J. Microbiol., 44: 1094-1101.

Felsenstein, J., 1993. PHYLIP (Phylogeny Inference Package). Version 3.5c, University of Washington, Seattle.

Hadrys, H., M. Balick and B. Schierwater, 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol., 1: 55-63.

Hernandez, G. and J. Olmos, 2004. Molecular identification of pathogenic and nonpathogenic strains of Vibrio harveyi using PCR and RAPD. Applied Microbiol. Biotechnol., 63: 722-727.

Hillis, D.M., C. Moritz and B.K. Mable, 1996. Molecular Systematics. 2nd Edn., Sinauer Associates, Sunderland, MA., USA., pp: 655.

Kiska, D.L. and P.H. Gilligan, 1999. Pseudomonas. In: Manual of Clinical Microbiology, Murray, P.R., E.J. Baron, M.A. Pfaller, F.C. Tenover and R.H. Yolken (Eds.). 7th Edn., ASM Press, Washington, DC., pp: 517-525.

Musa, N., L. Seong and W. Wee, 2008. Phenotypic and genotypic characteristics of *Vibrio harveyi* isolated from black tiger shrimp (*Penaeus monodon*). World Applied Sci. J., 3: 885-902.

Nei, M. and W.H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA., 76: 5269-5273.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89: 583-590.

Ortega-Rivas, A., J. Lorenzo-Morales, V. Alonso, N.J. Abreu, P. Foronda, A. Castillo and B. Valladares, 2003. Random amplified polymorphic dna profiles as a tool for the identification of *Acanthamoeba divionensis*. Curr. Microbiol., 47: 0084-0086.

Ortiz-Herrera, M., A. Geronimo-Gallegos, F. Cuevas-Schacht, L. Perez-Fernandez and R. Coria-Jimenez, 2004. RAPD-PCR characterization of pseudomonas aeruginosa strains obtained from cystic fibrosis patients. Salud Publica Mex., 46: 149-157.

Pujalte, M.J., A. Sitjà-Bobadilla, M.C. Macián, C. Belloch, P. Álvarez-Pellitero, J. Pérez-Sanchez, F. Uruburu and E. Garay, 2003. Virulence and molecular typing of *Vibrio harveyi* strains isolated from cultured dentex, gilthead, sea bream and European sea bass. Syst. Applied Microbiol., 26: 284-292.

Quednau, M., A. Siv, A.C. Petersson and G. Molin, 1997. Identification of clinically important species of *Enterococcus* Within 1 day with randomly amplified polymorphic DNA (RAPD). Curr. Microbiol., 36: 332-336.

Riley, L.W., 2004. Molecular Epidemiology of Infectious Disease. ASM Press, Washington DC.

Rowe, S.M., S. Miller and E.J. Sorscher, 2005. Cystic fibrosis. N. Engl. J. Med., 352: 1992-2001.

Sawabe, T., K. Kita-Tsukamoto and F.L. Thompson, 2007. Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. J. Bacteriol., 189: 7932-7936.

Shi, B. and X. Xia, 2005. Genetic variation in clones of Pseudomonas pseudoalcaligenes after ten months of selection in different thermal environments in the laboratory. Curr. Microbiol., 50: 238-245.

Sneath, P.H.A. and R.R. Sokal, 1973. Numerical Taxonomy. 1st Edn., W.H. Freeman and Co., San Francisco, USA., ISBN: 0716706970, pp: 573.

Sudheesh, P.S., K. Jie and H.S. Xu, 2002. Random amplified polymorphic DNA-PCR typing of *Vibrio parahaemolyticus* and *V. alginolyticus* isolated from cultured shrimps. Aquaculture, 207: 11-17.

Tenover, F.C., R.D. Arbeit and R.V. Goering, 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: A review for healthcare epidemiologists. Infect. Control Hosp. Epidemiol., 18: 426-439.

Thompson, F.L. and J. Swings, 2006. Taxonomy of Vibrios. In: The Biology of Vibrios, Thompson, F.L., B. Austin and J. Swings (Eds.). ASM Press, Washington, DC., pp: 29-43.

Van Belkum, A., 1994. DNA fingerprinting of medically important microorganisms by use of PCR. Clin. Microbiol. Rev., 7: 174-184.

Ventura, M., V. Meylan and R. Zink, 2003. Identification and tracing of *Bifidobacterium* species by use of enterobacterial repetitive intergenic consensus sequences. Applied Environ. Microbiol., 69: 4296-4301.

Walker, T.S., 1998. Microbiology. O.W.B. Saunders, Philadelphia, PA., pp: 173-181.

Welsh, J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Yeh, F.C., R.C. Yang and T. Boyle, 1999. POPGENE 32-version 1.31. Population Genetics Software. http.//www.ualberta.ca/~fyeh/fyeh/.